THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 26, Issue of September 15, pp. 15623-15631, 1990
Printed in U.S.A.

Transmembrane Movement of Heme*

(Received for publication, March 2, 1990)

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Evidence for CO-heme partitioning into and across lipid bilayers was obtained by kinetic and chromatographic studies. Biphasic time courses were observed when CO-heme was rapidly mixed with unilamellar lipid vesicles in a stopped-flow spectrometer. The initial rapid phase depended linearly on lipid concentration and was assigned to heme partitioning between the external solvent phase and the outer lipid layer of the membranes. The rate of the second, much slower phase was independent of both heme and lipid concentration. The fraction of absorbance change associated with this slower phase increased with increasing heme to lipid ratios and reached a maximum of ~45%. A similar slow phase was observed when membrane-bound heme was reacted with apomyoglobin. In the presence of excess globin, all of the CO-heme was extracted from the membranes to form native CO myoglobin. Under these conditions, the fractional amount of absorbance change associated with the slow dissociation phase was ~45%, regardless of the heme to lipid ratio. These results suggest strongly that the slow phases represent transmembrane movement of heme, from the outer to the inner lipid layer in the association reactions and from the inner to the outer layer in dissociation reactions.

The temperature dependence of the rate of CO-heme binding to the outer lipid layer was markedly different from that of transmembrane movement. The rate of the latter, slower process decreased greatly with increasing acyl chain length, whereas the rate of the initial binding process varied little with vesicle composition, as long as the membranes were examined above their melting temperatures. Finally, the two kinetically distinct bound heme fractions could be isolated directly by column chromatography.

Although *in vitro* combination of apomyoglobin and apohemoglobin with protoheme occurs readily, *in vivo* formation of the holoproteins is complicated by membrane barriers. Apoprotein is synthesized on the endoplasmic reticulum, whereas the terminal enzyme in the heme biosynthesis pathway, ferrochelatase, is located in the inner membrane of mitochondria (Granick and Beale, 1978; Taketani and Tokunaga, 1982; Daily and Fleming, 1983). The incorporation of iron into protoporphyrin IX occurs at an active site which faces the mitochondrial matrix (Harbin and Daily, 1985), and newly synthesized heme must pass through at least the inner and outer mitochondrial membranes before it can combine with apoprotein. Thus, movement of heme into and across lipid bilayers plays a crucial role in the formation of extramitochondrial heme proteins.

A number of investigators have shown that heme, in one form or another, readily associates with lipid bilayers (Tipping *et al.*, 1979; Ginsberg and Demel, 1983; Cannon *et al.*, 1984; Rose *et al.*, 1985). Most of these studies have used phosphatidylcholine liposomes as model membranes. The aromatic portion of heme is thought to intercalate between the acyl-chains of neighboring lecithins, and the propionate groups appear to be located in the polar region of the bilayer (Cannon *et al.*, 1984; Rose *et al.*, 1985).

In contrast to the equilibrium-binding experiments, measurements of the rate of heme transmembrane movement have been controversial (Cannon *et al.*, 1984; Rose *et al.*, 1985). Cannon and co-workers (1984) reported two greatly different rates (2 and 0.01 s^{-1}) for the efflux of hemin from lipid vesicles in the presence of apoproteins. These investigators concluded that the fast phase represented heme dissociation from the outer portion of the bilayer and that the slow phase represented transmembrane movement of hemin from the inner to the outer portion of the bilayer. They suggested that the slow rate of heme transbilayer movement was rate limiting for the dissociation of 30–50% of the bound heme molecules. Interpretation of these heterogenous time courses was complicated by the use of hemin which self-aggregates in aqueous solution and by evidence for slow iron chelation by endogenous lipid bases (Rose *et al.*, 1985). To avoid these complications, we examined the uptake and release of CO-heme, which is monomeric in dilute aqueous solutions (Smith, 1959; Light, 1987), and our initial kinetic and chromatographic experiments with small egg lecithin vesicles suggested that CO-heme dissociation from a bilayer was slower than transmembrane movement (Rose *et al.*, 1985).

In an attempt to resolve the apparent discrepancy between the results of Cannon *et al.* (1984) and Rose *et al.* (1985), we have carefully looked for slow phases in CO-heme uptake and release experiments with unilamellar membrane vesicles. The temperature dependence of the rates of the slow phases were compared with behavior observed for the transmembrane movement of lipid molecules. The gel filtration experiments described in Rose *et al.* (1985) were repeated with liposome vesicles of larger diameter (100 nm) prepared using an extrusion technique. In these new experiments, purified synthetic phosphatidylcholines were used instead of egg lecithin to reduce the possibility of heme binding to endogenous chelating ligands. Finally, a general mathematical model was developed to describe heme partitioning based on the assumption that the rate of transmembrane heme movement is slow relative to that for uptake and release by the outer membrane layer.

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§ Supported by United States Public Health Service Grant GM 35649 and by Grant C-612 from the Robert A. Welch Foundation.

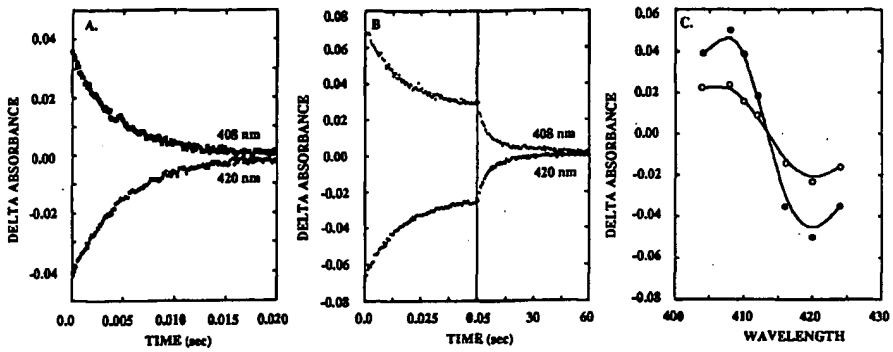

FIG. 1. **CO-heme uptake by lipsomes.** *A*, sample time courses for the reaction of 1 μM CO-heme with 300 μM egg lecithin liposomes. The association reaction was carried out in 0.05 M NaCl, 0.05 M Tris, pH 8.0, at 30 °C in the stopped-flow apparatus at 408 (*top trace*) and 420 nm (*bottom trace*). *B*, the reaction of 1 μM CO-heme with 50 μM DMPC/DCP (95:5) liposomes. The time courses were observed at 408 nm (*top trace*) and 420 nm (*bottom trace*). Note the two time scales. *C*, the wavelength dependence of the absorbance changes due to the observed fast (*closed circles*) and slow (*open circles*) phases for CO-heme binding to DMPC/DCP liposomes under the conditions in *panel B*.

MATERIALS AND METHODS[1]

RESULTS

CO-Heme Uptake Experiments—Sample time courses are shown in Fig. 1*A* for the association of CO-heme with large egg lecithin liposomes prepared by the extrusion method (see Miniprint). On the time scales examined, only a single phase was observed, regardless of conditions, and the fitted rate constant exhibited an almost linear dependence on lipid phosphate concentration which was similar to that reported by Rose *et al.* (1985). In contrast, markedly biphasic time courses were observed for the uptake of CO-heme by DMPC[2] containing liposomes (Fig. 1*B*). Both phases showed absorbance difference spectra which were characteristic of CO-heme binding to lipid (Figs. 1*C* and 2*S*).

The rate constant for the rapid kinetic phase observed for heme binding to DMPC vesicles exhibited a linear dependence on lipid phosphate concentration with an apparent bimolecular rate constant of $\sim 7 \times 10^5$ M^{-1} s^{-1} (Fig. 2*A*). A pseudo-first order rate constant equal to ~ 0.2 s^{-1} was observed for the slow phase and showed little or no dependence on phospholipid concentration (Fig. 2*A*). The fractional extent of the slow phase did depend markedly on the amount of lipid present, decreasing from ~45% at low concentrations of DMPC to ≤5% at high concentrations (Fig. 2*B*). Similar heterogeneous kinetic behavior was observed with 10 different liposome preparations containing DMPC, dimyristoyl phosphatidylglycerol, DCP, *cis*-Δ^9DMPC, dilauroyl phosphatidylcholine, DTPC, and DPDPC. Heterogeneous binding behavior was difficult to demonstrate in heme uptake experiments with longer acyl-chain lecithins on the same time scales.

The simplest explanations for the second phase seen with the DMPC vesicles are that there are two pools of aqueous CO-heme that react differently with the membranes or that CO-heme partitions into two distinct lipid phases at widely different rates. The first possibility, two free heme components, has been observed in studies of hemin binding to apohemoglobin (Gibson and Antonini, 1960). Solutions of hemin consist of monomers, dimers, and more complex aggregates and exhibit heterogeneous kinetic behavior (White, 1978). However, CO-heme appears to be monomeric at concentrations ≤10 μM (Smith, 1959; Light, 1987). If the dissociation of CO-heme aggregates caused the slow phase in uptake experiments, then the ratio of the fast to slow amplitudes should depend only on the initial free heme concentration and not that of the lipid. As shown in Fig. 2*B*, the fractional amount of slow phase decreased markedly with increasing lipid phosphate concentration.

Evidence for two distinct lipid compartments can be inferred from the data in Fig. 2. A simple model based on two independent lipid structures with different affinities for heme is unlikely since the rate for the second phase showed little dependence on lecithin concentration (Fig. 2*A*) and appears to be a true first order process. Two obvious lipid compartments are the outer leaflet of lecithin molecules arranged with their polar head groups oriented toward the bulk solvent and the inner leaflet with polar head groups facing the inner aqueous phase. Initially, free heme can only bind to the outer side of the bilayer. When the outer portion of the membrane is saturated with heme, further binding will occur only after net movement of heme from the outer to the inner portion of the bilayer. If the rate of this transbilayer movement is faster than the initial binding process, then distinction between the two sides of the bilayer is unnecessary, only one lipid pool needs to be considered, and a monophasic time course would be expected. If the rate of heme crossing from one side of the membrane to the other is slow as suggested by Cannon *et al.* (1984), then heme binding to the outer bilayer and heme "flipping" across the membrane would become distinct physical events, and the two processes should exhibit second order and first order reaction mechanisms, respectively. The results in Figs. 1 and 2 for DMPC vesicles suggest that transmembrane movement is slow and distinct from the initial binding process.

CO-Heme Release Experiments—If the transmembrane movement of heme is slow, then this process should also be observed when measuring the dissociation of CO-heme from liposomes. As shown in Fig. 3*A*, biphasic time courses were observed when liposomes containing short-chain phosphatidyl cholines and CO-heme were mixed with apomyoglobin.

[1] Portions of this paper (including "Materials and Methods," Figs. 1S and 2S, and Equations 1S–19S) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: DMPC, dimyristoylphosphatidylcholine; DCP, dicetylphosphate; DTPC, ditridecanoylphosphatidylcholine; *cis*-Δ^9DMPC, dimyristoleoyl phosphatidyl choline (9-cis-tetradecanoic acid); DPDPC, dipentadecanoylphosphatidylcholine. A list of the T_m values for the lecithins is given in Table I of Light and Olson (accompanying article).

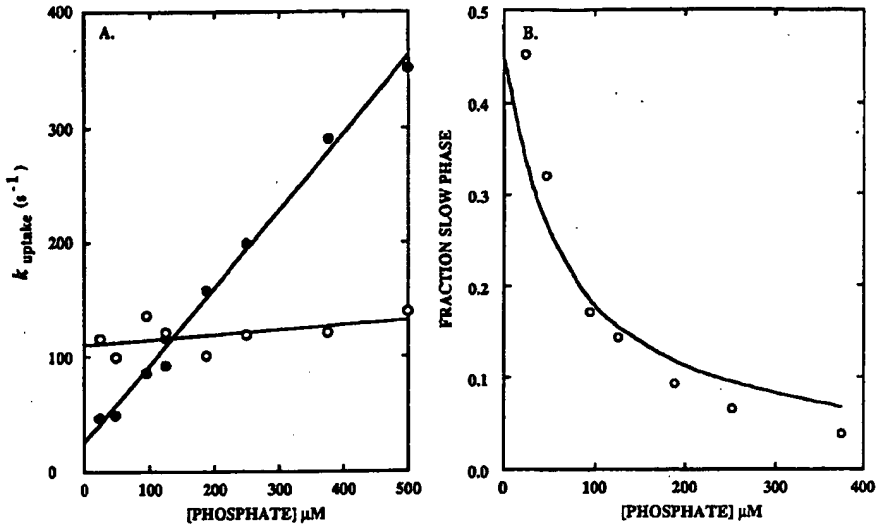

FIG. 2. **The phospholipid concentration dependence of the rates of CO-heme uptake by DMPC/DCP (95:5) liposomes.** The reaction of 2 μM CO-heme with DMPC/DCP (95:5) liposomes at 30 °C was observed at 420 nm in the stopped-flow apparatus. *A*, the dependence of the observed pseudo-first order rate constants (s^{-1}) for the fast (*closed circles*) and slow ($k_{uptake, slow} \times 10^3$, *open circles*) phases. *B*, the observed dependence of the fractional amount of the slow absorbance change (*open circles*) and the theoretical dependence based on Equation 2 (*line*, calculated assuming $KpV_L = 0.03\ \mu M^{-1}$).

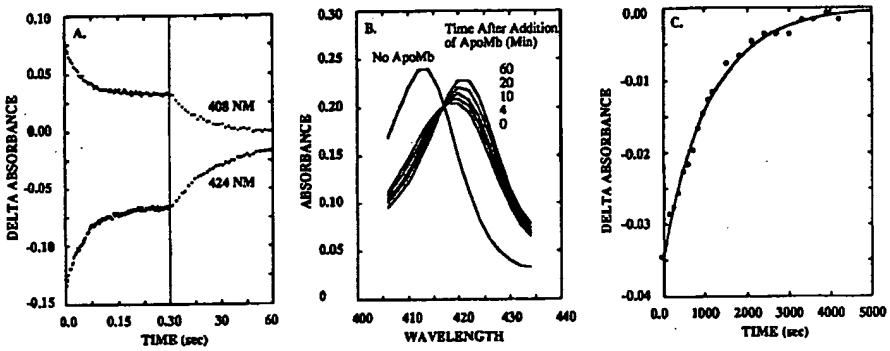

FIG. 3. **CO-heme release from liposomes.** *A*, the reaction of 12 μM apomyoglobin with 100 μM *cis*-Δ^9DMPC/DCP (9:1) liposomes that had been preincubated with 1 μM CO-heme. The dissociation reaction was carried out in 0.05 M NaCl, 0.05 M Tris, pH 8.0, at 30 °C in the stopped-flow apparatus. The time courses were observed at 424 nm (*closed circles*) and 408 nm (*open circles*). Note the two time scales. *B*, time resolved spectra for the dissociation of CO-heme from egg lecithin vesicles. The spectra were measured after the addition of 12 μM apomyoglobin to a cuvette of premixed 2 μM CO-heme and egg lecithin vesicles (100 μM phosphate). *C*, reaction time course for CO-heme dissociation from egg lecithin vesicles which constructed by plotting the change at 424 nm as a function of time. The *closed circles* represent the measured data points and the *solid line* represents a fit to a single exponential expression.

The two phases exhibited similar wavelength dependences, and the rates of the slow phases matched those of the slow first order processes measured for the uptake of heme by the same set of liposomes (Table I). Rose *et al.* (1985) have shown that the fast phase represents the dissociation of heme from the outer lipid layer ($t_{1/2}$ = 0.05–0.20 s).

A complete analysis of these results is presented under the "Discussion" and in the Miniprint. The rates for the slow phases decreased markedly with increasing acyl chain length of the lecithins being studied (Table I, see also Fig. 6). These results imply that Rose *et al.* (1985) failed to observe slow phases for long acyl chain egg lecithin vesicles, not because the rates were too fast, but rather because they were too slow to detect easily in stopped-flow, rapid mixing experiments. This idea was tested by mixing long-chain, unsaturated lecithin vesicles containing CO-heme with apomyoglobin and following heme dissociation for long times in a spectrophotometer (Fig. 3, *B* and *C*). Approximately 50% of the CO-heme efflux was rapid. The remaining absorbance changes had an isosbestic point at 418 nm and negative and positive difference peaks at 414 nm and 424 mn, respectively, indicating that CO-myoglobin was still being formed, albeit very slowly (Fig. 3*B*). When CO-heme and apomyoglobin were mixed prior to the additions of liposomes, or liposomes were mixed with apomyoglobin prior to the addition of CO-heme, no slow absorbance changes were observed. These controls indicated that the extremely slow absorbance changes shown in Fig. 3*B* were due to heme dissociating from the liposomes and not from interactions between reconstituted myoglobin and the membranes. Reaction time courses were constructed by plotting the absorbance change at a single wavelength as a function of time (Fig. 3*C*). The transmembrane rates for several long-chain phosphatidyl cholines were determined by this method and are listed in Table I.

The Temperature Dependence of Transmembrane Movement—The temperature dependence of the rate of the slow phase associated with CO-heme uptake by DMPC liposomes exhibited non-Arrhenius behavior in the region near the lipid phase transition temperature (T_m). A peak centered around the T_m value (23.8 °C, Miniprint) was observed in a plot of log k_{slow} *versus* $1/T$ (Fig. 4). When *cis*-Δ^9DMPC, (T_m < 0 °C; Small, 1986) was used instead of DMPC, simple Arrhenius behavior was observed (Fig. 4). The slow rate for all saturated DMPC-containing liposomes showed temperature depend-

TABLE I

Rates of slow phases observed in CO-heme uptake and release experiments in 0.05 M NaCl, 0.05 M Tris, pH 8.0

The abbreviations used are: POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; OSPC, 1-oleoyl-2-stearoyl phosphatidylcholine.

Liposome composition	Temperature	$k_{slow,\ uptake}$	$k_{slow,\ release}$
	°C	s^{-1}	s^{-1}
DLPC	20	0.36	0.087
	30	0.52	0.19
DTPC	30	0.40	0.083
cis-Δ^9DMPC	30	0.040	0.038
cis-Δ^9DMPC/DCP (9:1)	20	0.022	0.018
cis-Δ^9DMPC/DCP (9:1)	30	0.13	0.048
DMPC	34	0.15	
DMPC/DCP (95.5)	30	0.11	
DMPC/DCP (90:10)	30	0.11	
DMPC/DCP (85:15)	30	0.13	
DPDPC	30	0.044	0.057
cis-Δ^9DPPC/DCP (9:1)	30		0.0055[a]
POPC/DCP (9:1)	30		0.0025[a]
OSPC/DCP (9:1)	30		0.00085[a]
Egg lecithin	30		0.00083[a]

[a] These rates were measured with a spectrophotometer as described in the Miniprint and Figs. 3*b* and *c*.

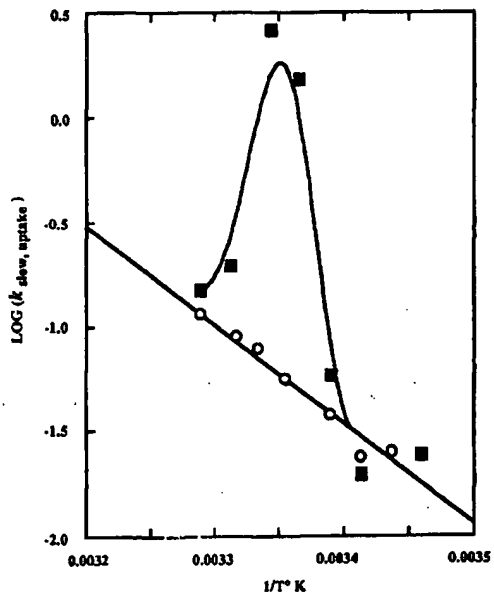

FIG. 4. **The temperature dependence of the rate of the slow phase observed for CO-heme uptake by liposomes.** The *closed squares* and *open circles* represent Arrhenius plots of the $k_{slow,\ uptake}$ values (s^{-1}) for CO-heme uptake by 125 μM DMPC/DCP (95:5) and 95 μM *cis*-Δ^9 DMPC/DCP (9:1) liposomes, respectively. The *solid straight line* represents a linear fit of the rate data for *cis*-Δ^9 DMPC/DCP (9:1) liposomes. The *curved line* was drawn through the data for DMPC/DCP (95:5) vesicles and is not theoretical.

ences with peaks around 25 °C. The width of the Arrhenius peak closely matched that of the gel to liquid crystalline phase transition peak of DMPC/DCP (9:1) obtained by differential scanning calorimetry (Fig. 1*S*). Non-Arrhenius behavior, but with different peak positions, was also obtained for the rates of slow phases measured in CO-heme uptake experiments with DTPC and DPDPC liposomes (Light, 1987), which have phase transitions at 15 and 34 °C, respectively (Small, 1986). Similar temperature dependences were observed for the slow phase of heme efflux in the presence of excess apomyoglobin.

Using NMR spectroscopy, de Kruijff and Van Zoelen (1978) observed that the rate of phospholipid transmembrane movement increased markedly in the temperature region of the gel to liquid crystalline transition. An Arrhenius plot of their data is very similar to that shown in Fig. 4 for the slow rate of heme uptake by DMPC vesicles. Marked rate increases at the phase transition temperature have also been observed in bilayer permeability studies (Papahadjopoulos *et al.*, 1973), and these phenomena were explained by an increase in lateral compressibility due to the coexistence of gel and liquid-crystalline lipid. Thus, the anomalous temperature dependence of the slow process in CO-heme uptake further supports the idea that this kinetic phase represents transmembrane movement of porphyrin molecules.

Apparent activation energies for the slow phase in CO-heme uptake experiments were measured in the 15–30 °C range for liposomes which did not have T_m values in this region. The E_a values for this transmembrane process varied widely depending on lipid composition: 6.0, 22, and 30 kcal/mol for DLPC, *cis*-Δ^9DMPC/DCP, and DPDPC vesicles, respectively. The increase in E_a with increasing acyl-chain length correlates with the decrease in the overall rate constant for transmembrane movement (Fig. 6). The activation energy required for transmembrane migration of DMPC in DMPC vesicles at temperatures above 25 °C was measured to be 24.7 kcal/mol (de Kruijff and Van Zoelen, 1978), which is similar to that for heme flipping in liposomes containing C_{14} acyl lecithins. Homan and Pownall (1988) measured activation energies in the range 25–35 kcal/mol for the transmembrane movement of fluorescent-labeled phosphatidylcholines, glycerols, and ethanolamines in sonicated DMPC vesicles at temperatures greater than 30 °C. In all cases, the rates for phospholipid flipping were on the order of 10^{-5} to 10^{-7} s^{-1}, which is roughly 1000-fold slower than the rate for transmembrane movement of heme (Table I).

Chromatographic Analysis of Heme Transmembrane Movement—The experimental observations presented in this paper are consistent with the model first suggested by Cannon *et al.* (1984) in which heme transmembrane movement is slow. They contradict our earlier chromatographic experiments (Rose *et al.*, 1985) in which heme bound to egg lecithin was mixed with excess apohemoglobin and the products separated by Sephadex G-200 chromatography. All of the heme appeared to be taken up by the globin before the protein was separated from the liposomes. At the time, we concluded that the rate of heme movement from the inner to the outer portion of the membrane must have been rapid for all of the heme to be available to the apoprotein. However, if the measured rates for the slow phases seen in Figs. 1–3 are an indication of the rate of heme transmembrane movement, then the half-time for heme flipping across egg lecithin membranes should be approximately 10 min, and it should have been possible to separate the liposomes from the apomyoglobin before all the heme had time to cross the membrane and form reconstituted myoglobin.

In the experiment shown in Fig. 5, large unilamellar egg lecithin liposomes containing bound CO-heme were loaded onto a Sephadex G-200 column immediately after mixing with apoprotein. The lipid vesicles eluted as a single peak, but the heme eluted in two fractions. The first heme peak comigrated with the liposomes, and the second peak eluted as newly reconstituted CO-myoglobin. Thus, not all of the bound CO-heme was available to the apomyoglobin during the time it took to separate the liposomes from the protein. At 25 °C, less than 30% of the total heme comigrated with the liposomes,

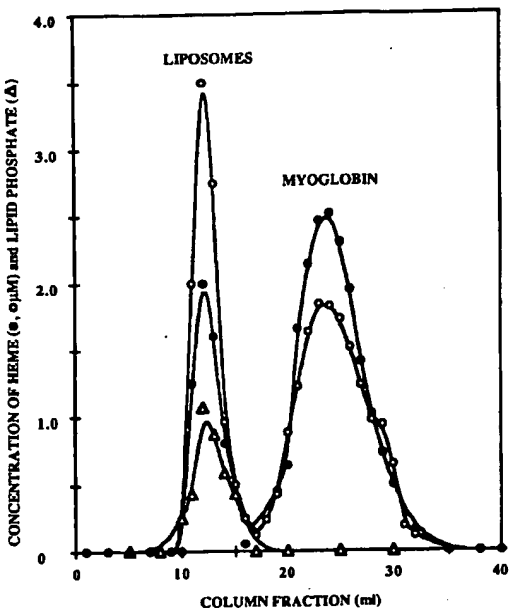

FIG. 5. **Separation of newly reconstituted myoglobin and egg lecithin vesicles by Sephadex G-200 chromatography.** Liposome suspensions containing 10 mM phospholipid in 0.05 M NaCl, 0.05 M Tris, pH 8.0, were equilibrated with 125 μM CO-heme, mixed rapidly with a 2-fold excess of apomyoglobin, and then quickly loaded onto a Sephadex G-200 column. The eluting buffer and column had been equilibrated with CO, and sodium dithionite was present to keep the heme reduced during the mixing with the egg lecithin vesicles and apomyoglobin. The *open diamonds* represent the lipid phosphate concentration in the fractions for the 25 °C experiment, and the *open* and *closed circles* represent the measured heme concentrations of the experiments at 4 and 25 °C, respectively.

whereas the rest formed myoglobin. At 4 °C, more than 40% of the total heme remained membrane-bound. When the CO-heme-liposome-globin mixture was allowed to incubate for 30–60 min before separation, all of the heme eluted as myoglobin.

Similar experiments were carried out with hemin and vesicles composed of POPC or DTPC (data not shown). All of the DTPC-bound hemin, but only a portion of the POPC-bound hemin was taken up by apomyoglobin during the course of the separation. These results correlate with the rates of the slow phases observed for both of these lipids in CO-heme uptake reactions (Table I). The measured rate constant of the slow phase seen for DTPC liposomes is 0.083 s^{-1} and indicates a half-time of only 8.3 s. This is much shorter than the half-time of the slow phase for POPC vesicles (~5 min) and the time that it takes to separate the liposomes from the protein on the column (again, ~5 min).

DISCUSSION

Four separate experimental observations indicate that heme transmembrane movement is a slow, kinetically distinct process: 1) similar slow kinetic phases were observed during CO-heme uptake and release by lipid bilayers, 2) the rates of the slow phases were markedly dependent on the acyl-chain length of the phospholipid membrane, 3) the temperature dependences of the rates of the slow phases resemble those observed for other transmembrane movements, and 4) two kinetically distinct, membrane-bound heme fractions can be detected directly by column chromatography. Taken together these results suggest strongly that three steps are needed to describe heme binding to a lipid membrane: heme partitioning into the outer layer, transmembrane movement, and partitioning into the inner aqueous phase which is encapsulated by the liposome. Mechanistically, this can be described as

$$H_a^o \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H_m^o \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H_m^i \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} H_a^i \quad (1)$$

Where H_a^o represents free CO-heme in the outer aqueous layer; H_m^o, bound heme in the outer membrane lipid layer; H_m^i, bound heme in the inner membrane lipid layer; and H_a^i, free heme in the inner aqueous layer. k_1 and k_{-1} represent the first order rate constants for partitioning into and out of the membrane layers, and k_2 and k_{-2}, the rate constants for transmembrane movements. Derivations of the rate and equilibrium equations for heme uptake, release, and the corresponding, slow transmembrane phases are presented in the Miniprint.

Interpretation of Heme Uptake and Release Kinetics—For liposomes with a radius of 50 nm and a bilayer thickness of 4.5 nm, the fractional amount of absorbance change for slow transmembrane movement in CO-heme uptake reactions is given by:

$$f_{\text{slow, uptake}} = \frac{0.45}{1 + K_p V_L C_L\,(0.55)} \quad (2)$$

where 0.45 and 0.55 are the fractional volumes of lipid present in the inner and outer membrane layers, respectively; K_p is the heme partition constant for going from an aqueous to a lipid phase (k_1/k_{-1} in Equation 1); and $V_L C_L$ is the total volume fraction of lipid in the aqueous suspensions (see Miniprint, Equations 5S–11S). At low lipid to heme ratios (*i.e.* $C_L \rightarrow 0$), $f_{\text{slow, uptake}}$ should approach 0.45 since the outer layer quickly becomes saturated with heme and further net binding cannot occur until equilibration with the inner lipid phase. At high lipid concentrations, $f_{\text{slow, uptake}}$ approaches 0 since the volume of outer lipid phase is sufficient to bind all the available heme, and as a result, no net absorbance changes are associated with transmembrane movement. This dependence is observed experimentally as shown in Fig. 2*B* where the *solid line* represents a fit to Equation 2.

The rate of the slow, transmembrane phase obtained in heme uptake experiments with large liposomes (R_0 = 50 nm) is both observed and predicted to show little dependence on lipid phosphate concentration (Fig. 2*A* and Equations 13S and 14S, Miniprint).

$$k_{\text{slow, uptake}} = \frac{k_2 K_p V_L C_L\,(0.45)}{1 + K_p V_L C_L\,(0.55)} + k_{-2} \quad (3)$$

Since for large liposomes the rate of flipping is expected to be independent of direction in the absence of a membrane potential, k_2 should equal k_{-2}, and the maximum change in rate is expected to be less than 2-fold (k_2 at $C_L = 0$ and $1.82k_2$ at $C_L \rightarrow \infty$).

In release experiments, apomyoglobin is mixed with liposomes preincubated with CO-heme. Under these conditions, the heme is initially distributed between the outer and inner lipid layers according to their relative volumes. In the presence of excess globin, the fraction of slow absorbance change associated with transmembrane movement is both observed and expected to be ~0.45, regardless of the heme to lipid ratio (Fig. 3, and Miniprint). In release experiments at high globin concentrations, the observed rate of transmembrane movement is equal to k_{-2} alone because as soon as the heme group flips to the outer layer it is incorporated irreversibly into myoglobin (Equation 17S, Miniprint). Thus, the rates of the slow, transmembrane phase observed in heme release experiments with apomyoglobin should be less than those observed in the corresponding heme uptake reactions where the rate of equilibration across the membrane is being measured. The

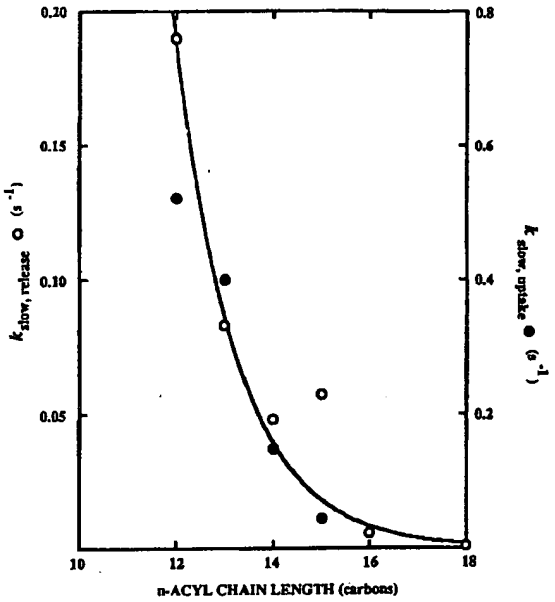

FIG. 6. **Rate constants for the slow phases as a function of acyl-chain length at 30 °C.** The first order rate constants (s^{-1}) for the slow phases were measured in either uptake (*solid circles*) or release (*open circles*) experiments as indicated in Table I. The curve was drawn through the points using an exponential function but is not theoretical.

results in Table I and Fig. 6 confirm this prediction, although the uncertainties in the rate constants preclude a quantitative analysis.

Previous Observations—The results and analyses presented in Figs. 1–6 support strongly, if not unequivocally, Cannon *et al.*'s (1984) conclusions that transmembrane movement of heme is slow. In our earlier work, we were incorrect in assuming that this process was too rapid to measure (Rose *et al.*, 1985). There were three problems with our previous experiments. First, the egg lecithin liposomes were prepared by the ethanol injection method of Batzri and Korn (1973) and had an average size of 20 nm in diameter. As a result, the fractional lipid volume of the inner layer of these much smaller vesicles was only 0.23 ($(R_i^2/(R_o^2 + R_i^2)$ where $R_o = 10$ nm and $R_i = 5.5$ nm). Thus, even in release experiments where the CO-heme was pre-equilibrated with the vesicles, only 23% of the total absorbance change could be associated with flipping. This accessible inner volume was probably further reduced by tight packing of the polar head groups due to the large radius of curvature for the inner layer of small vesicles (Cornell *et al.*, 1980). Wimley and Thompson (1990) have shown that this curvature effect markedly slows the rate of transmembrane movement of DMPC in small sonicated liposomes. Second, as shown in Table I, the rate of transmembrane movement is very slow for egg lecithin vesicles ($t_{1/4} \cong 10$ min), and we did not look for phases on such long time scales. Third, although the rate of flipping is slow relative to that of most reactions examined by rapid mixing techniques, this process does occur quickly enough to interfere with separations carried out chromatographically ($t_{1/4} \cong 5$–20 min). In our latest work, greater care was taken to ensure that the time interval between mixing apomyoglobin with heme-containing vesicles and application to the gel filtration column was as small as possible (≤30 s), and that short, rapidly flowing columns were used.

Dependence on Acyl Chain Length and Physiological Significance—The rate of transmembrane CO-heme movement at 30 °C depends markedly on the acyl chain length of the lecithin molecules; the longer the chain length, the slower the rate of flipping measured in either association of dissociation reactions (Fig. 6). This result implies that the observed rate depends inversely on the distance traversed by the charged propionates and agrees with the idea that the major barrier for phospholipid flipping is movement of the polar head groups through the apolar region of the bilayer (Homan and Pownall, 1988; Wimley and Thompson, 1990).

The unusual increase in the rate of heme flipping observed at the phase transition temperature (Fig. 5) indicates that the state of the liposome plays a significant role in transmembrane movement. At the T_m, the lipid population is equally divided between states with sufficient energy to be liquid and those cold enough to be gel-like. Continuous packing is maintained in liquid-crystal phases because the neighboring acyl chains fill gaps created by thermal fluctuations. In the gel phase, packing is even closer because of reduced motion. However, at the interface of the two phases, gaps may be caused by thermal motion of liquid acyl chains that are not compensated for by neighboring gel state molecules. Thus, although the net fluidity of the liposome decreases as the temperature is lowered through the T_m region, large discontinuities occur at the interfaces of the gel and liquid phases. The number of these gaps is maximal at the T_m which accounts for the large increase in the rates of transmembrane movement of heme (Fig. 5), protons (Papahadjopoulos *et al.*, 1973), and lipid molecules at this temperature (De Kruijff and Van Zoelen, 1978).

The transmembrane movement of CO-heme in liposomes containing long, unsaturated acyl-chains is very slow ($k_2 \approx 0.001\ s^{-1}$; last two rows, Table I). If this value is an indication of the *in vivo* flipping rate, then the time required for heme to travel from the inner matrix of the mitochondria through the inner and outer mitochondrial membranes could limit the cytoplasmic rate of heme protein formation. However, the rate constants measured in these model membrane systems may be lower than those *in vivo*. First, the results of Cannon *et al.* (1984) suggest that the rate of transmembrane movement of hemin may be 4–10-fold greater than that of CO-heme under comparable conditions; and second, rate enhancement, similar to that observed *in vitro* at temperatures near the phase transition temperature, might be caused by proteins or other factors that create discontinuities in the apolar regions of the membranes. In addition, the half-time of 10 min measured for heme flipping in egg lecithin liposomes at 30 °C may not be all that large with respect to the times required for protein synthesis since the estimated time for complete formation of a hemoglobin α-chain in reticulocytes is ≈ 3 min at 37 °C (Lehninger, 1975). Finally, the slow rate of heme flipping could be advantageous. If there were no kinetic barriers to movement through membranes, heme would rapidly disperse within cells and be lost to the surroundings with possible toxic consequences to neighboring tissue.

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SUPPLEMENTAL MATERIAL

to

TRANSMEMBRANE MOVEMENT OF HEME

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I. MATERIALS AND METHODS

A. Liposome Preparation and Characterization:

The extrusion method - Dicetyl phosphate and cholesterol were purchased from Sigma. All other lipids were purchased from Avanti Polar-Lipids, Inc. The purity of these compounds was checked by TLC on silica gel plates. The phospholipid content of the unilamellar vesicles was assayed by the method of Chen *et al* (1956).

A series of liposomes of defined size but varying composition were prepared by rapid extrusion (Hope *et al.*, 1985; Mayer *et al.*, 1986). The lipid components were weighed and mixed in a minimum amount of chloroform/methanol 2:1 in a round bottom flask. A Büchler rotoevaporator was used to remove the chloroform/methanol at 36°C and to deposit the lipid mixture as a thin white film along the sides of the flask. Large multilamellar vesicles were prepared by adding tris buffer (0.05 M NaCl, 0.05 M Tris, pH 8.0) to the dry lipid and vortexing. A typical total lipid concentration was 25 mM. The aqueous lipid dispersion was then forced through polycarbonate filters (Nucleopore Corp.) using the Extruder™ (Lipex Biomembranes, Inc.). 2-8 ml of sample was put into the central cavity of the apparatus through a quick-release valve and then gas pressure was applied. The only exit for the sample was through the polycarbonate filters located at the bottom of the chamber. Pressures of up to 800 lb/in^2 were employed and required two stacked filters to prevent tearing. The cycle was repeated for a total of at least 8 passes to insure uniformity of the sample (see Light, 1987).

Multilamellar vesicles were still present in the effluent from filters with pore sizes larger than 200 nm (Mayer *et al.*, 1986). A final pore size of 100 nm was routinely used to insure that only unilamellar liposomes were present. To prevent the filters from clogging, especially in mixtures containing cholesterol, extrusion was started at a large pore size, generally 500 nm, and then decreased by changing the filters. All manipulations, including the original dispersion step, were carried out at a temperature greater than the phase transition temperature of the lipid mixture by immersing the Extruder™ in a water bath.

Electron microscopy - The structure and size range of the vesicles were examined by freeze-fracture and cryotransmission electron microscopy with the assistance of Dr. Ron Price at the Naval Research Laboratory, Bethesda, MD. The electron micrographs were taken on a transmission electron microscope (a Carl Zeiss EM10-CA with a CryoStage). For freeze fracture, the samples were transferred to Balzers copper specimen plates (Hudson, NH), blotted to remove excess fluid, equilibrated at room temperature, and then quickly frozen by plunging into a nitrogen crystallizer. After freezing, the samples were placed in a BAF 400D freeze fracture device, fractured, and replicated at -100°C and 10-6 Torr. The replicas were formed with 2 nm Pt-C and 20 nm of carbon film, floated off into distilled water, exposed to sodium hypochlorite for 3 hours, rinsed in distilled water, cleaned with 20% ethanol for an hour, and then transferred to Butvar B-98 coated grids for examination.

Cryo-TEM was performed by putting a minimal amount of sample directly on a Butvar B-98 coated grid, blotting off the excess fluid, and then rapidly freezing the specimen by plunging the grid into a nitrogen slurry. The sample was then transferred to a CryoStage in liquid nitrogen and placed in the electron microscope, taking care not to allow the sample temperature to rise above the devitrification temperature (140 K). The observation temperature was typically below 115°K and low electron doses were used to preserve the specimen's structure. The presence of vitreous instead of hexagonal ice was checked by electron diffraction. The presence of any internal structures in the liposomes was probed for by altering the focus through the liposome.

No evidence was found for internal liposomal structures in DMPC, DMPC/DCP (9:1), or DMPC/Chol (6:4) vesicles when the focus of the electron microscope was moved through liposomes that had been rapidly frozen with a nitrogen slurry. If multilamellar structures had been present they would have been seen as bilayers within bilayers. DMPC/Chol (6:4) vesicles did have a tendency to aggregate, whereas the DMPC/DCP (9:1) liposomes were almost never seen touching. The addition of hemin to DMPC vesicles at high heme to phosphate ratios (1:2) appeared to promote the formation of smaller structures as well as to promote aggregation.

DMPC vesicles were also examined by freeze fracture techniques, both in the absence and presence of low levels of hemin. The vesicles appeared to be relatively homogeneous with an average diameter of 100 nm. Again, there was no evidence of internal structures, the fractures were clear depressions or bulges without any signs of a secondary fracture. The DMPC vesicles were subjected to a freeze etching procedure designed to highlight any features that might be particularly deep, but again, the vesicles appeared to be simple unilamellar spheres, with a diameter of 100 nm when prepared by extrusion with filters containing 100 nm pores (see Light, 1987).

Differential scanning calorimetry - One possible consequence of heme binding to membranes is an alteration in the lipid bilayer structure. This possibility was tested by examining the gel to liquid crystalline phase transition of DMPC liposome/CO-heme mixtures using a Microcal MC-2 differential scanning calorimeter that was interfaced to an IBM PC with a DA-2 data aquisition and analysis system (Microcal). The scan rate was 30°C per hour with a 15 second filter constant. Concentrated liposome suspensions (5-20 mM phosphate) were used. The data were analyzed in terms of phosphate content, peak height, location, width, and area. These measurements were made with the help of Dr. Martha Mims at the Baylor College of Medicine, Department of Medicine, Houston, Texas.

The main transition temperature for DMPC liposomes prepared by the extrusion method was 23.8°C (Figure 1S). Our measured T_m agrees well with literature values of 23.9, 23.6, and 23.8°C (Mabrey and Sturtevant, 1976; Chen *et al.*, 1980; Huang *et al.*, 1982, respectively). It should be pointed out that these values are significantly higher than those obtained for DMPC liposomes made by sonication (18°C, Melchoir and Steim, 1976). The phase transition of small unilamellar vesicles is 5°C lower than that of their larger counterparts and the transition itself occurs over a broader temperature range. These differences between sonicated and extruded vesicles of the same lipid composition reflect a difference in chain packing induced by the considerable degree of curvature in the much smaller sonicated particles (Mabrey and Sturtevant, 1978; Cornell *et al.*, 1980; Brouillette *et al.*, 1982). The enthalpy change measured for our large DMPC vesicles, 5.7 kcal/mol, also agrees with literature values of 5.44, 6.26, and 5.4 kcal/mol for DPMC liposomes (Mabrey and Sturtevant, 1976; Mason and Huang, 1981; Huang *et al.*, 1982, respectively).

The measured T_m for DMPC/DCP (90:10) liposomes was 26.3°C (Fig. 1S). Only one peak was observed, indicating that the lipids are miscible, and the enthalpy changes for the two liposome preparations (DMPC and DMPC/DCP (90:10)) were similar, 5.7 kcal/mole vs. 6.7 kcal/mole, respectively. These results suggest that the peak shift comes from the cooperative interactions between the two lipids and not from two separate lipid pools. For a similar amount of heme (10%) in DMPC liposomes, the T_m was only slightly affected (23.3°C, Fig. 1S), and there was little change in ΔH (5.6 kcal/mol). These data provide direct evidence that the lipid bilayer structure is not radically altered by low levels of heme binding.

B. Kinetic Measurements:

Stopped flow, rapid mixing experiments were carried out in a Gibson-Durrum apparatus interfaced to microcomputer using OLIS, Inc. software and hardware (see Rose *et al.*, 1985). The various types of CO-heme absorbance changes that were followed are shown in Fig. 2S. An IBM 9430 spectrophotometer with automated data collection routines was used when following extremely slow reactions ($t_{1/2} \geq 10$ min.). Absorbances at twenty discrete wavelengths were collected simultaneously at various time intervals and then plotted as time resolved spectra. In this manner, either changes at a particular wavelength could be monitored to obtain rate constants, or the whole spectrum could be examined qualitatively. The efflux of heme from liposomes into the aqueous surroundings was measured directly by following the incorporation of CO-heme into apomyoglobin (Cannon *et al.*, 1984; Rose *et al.*, 1985).

In all of the kinetic experiments, the reactant solutions were purged with 1 atm of CO to remove O_2, and then small amounts of sodium dithionite (0.1 to 1.0 mM) were added to the heme, liposome, and apomyoglobin solutions to ensure complete anaerobiosis. The solutions were kept in stoppered 5-20 ml syringes, sealed cuvettes, or tonometers, and additions of reagents were made with gas-tight Hamilton syringes. Under these conditions, no oxidation or degradation of heme was detected, as monitored by heme

absorbance in the Soret wavelength region. The expected total absorbance changes, wavelength independent time courses, and isosbestic points were observed, even for very slow CO-heme efflux reactions (Figs. 1 and 3, Main Text). A complete description of these procedures and potential problems due to heme oxidation in liposome suspensions has been presented by Light (1987) and Rose *et al.*, (1985).

In kinetic experiments, slow, wavelength independent changes were observed when high lipid concentrations were mixed with CO-heme. Solutions premixed with heme and then mixed with buffer exhibited similar behavior. A number of reports indicate that liposomes can fuse together to form larger vesicles (Schultery *et al.*, 1980; Gibson and Strauss, 1984; and Morris *et al.*, 1985). Exposure to low temperatures promotes aggregation of vesicles with a high phase transition temperature. DLPC, DMPC, and DPDPC vesicles precipitated out of suspension if left overnight in the refrigerator, whereas *cis* Δ^9 DMPC vesicles were stable under similar conditions, presumably because of their much lower phase transition temperature.

To reduce these complications, experiments were carried out at reduced liposome and heme concentrations. However, the lipid phosphate concentration had to be high enough for significant heme binding. A further precaution was the addition of DCP or DMPG to many of the lipid mixtures. Electrostatic repulsion between negatively charged liposomes was sufficient to keep DMPC/DCP (9:1) vesicles from aggregating overnight at 4°C, even though the T_m of these vesicles is 23-26°C.

C. Column Chromatography:

Molecular sieve chromatography was used to separate liposomes from myoglobin in heme release experiments. This allowed a determination of the fraction of the lipid-bound heme that was available for rapid uptake by the apomyoglobin. Separations were carried out with a 2 x 20 cm Sephadex G-200 column at both 4 and 25°C and under anaerobic conditions using CO-equilibrated buffer containing sodium dithionite. Every effort was made to minimize the time between mixing apoprotein with the heme containing liposomes, loading the mixture on the column, and separating the protein from the lipid material. Fractions were collected at a rate of 30 ml/hr and the absorbance of the effluent at 280 nm was monitored continuously. Lipid content of the effluent was analyzed by phosphate assay.

The column was monitored visually, and spectra of samples that appeared to contain the heme were measured between 350 and 450 nm. The presence of CO-heme was obvious by its red color (or green for hemin) which was quite different from the milky white appearance of liposomes. The concentrations of free CO-heme and hemin were determined from the absorbances at 407 nm and 390, respectively. The liposome absorbance contribution was estimated from the wavelength dependence of the light scattering observed for large vesicles alone (Light, 1987). The heme content of the myoglobin fractions were calculated using extinction coefficients of 157 $cm^{-1}mM^{-1}$ at 409 nm and 187 $cm^{-1}mM^{-1}$ at 423 nm for metmyoglobin and CO-myoglobin, respectively (Antonini and Brunori, 1971).

In CO-heme efflux experiments, there was no loss of heme pigments as long as the initial incubation mixture was kept oxygen free and the column was completely sealed and pre-equilibrated with anaerobic buffer containing CO and sodium dithionite (0.1 to 1.0 mM). CO-heme is relatively stable in liposomes and is well protected from degradation in myoglobin. The fractions containing liposomes and CO-heme were usually allowed to oxidize completely to hemin after eluting from the column to prevent measurements of multiple heme species. The myoglobin fractions were measured as the reduced CO form. No residual heme pigments remained on the columns which were completely clear after elution of myoglobin.

II. ANALYSIS OF HEME UPTAKE, RELEASE, AND TRANSMEMBRANE MOVEMENT

In our previous work, we developed a general model for heme partitioning between buffer and the lipid matrix of membrane vesicles (Rose *et al.*, 1983). The equilibrium between aqueous and membrane-bound heme was described by a partition constant, K_p:

$$K_p = H_m/H_a \tag{1S}$$

where H_m and H_a are the heme concentrations in the membrane and aqueous phases, respectively. The fractional amount of heme bound in the lipid phase is obtained by consideration of the volume fractions of the two phases and the total solution concentration of heme, H_t:

$$H_t = H_b + H_f \tag{2S}$$

$$H_t = H_m V_L C_L + H_a (1 - V_L C_L) \tag{3S}$$

where H_b and H_f are the total solution concentrations of lipid-bound and free aqueous heme, respectively. V_L and C_L are the partial molar volume of phosphatidyl choline or lipid, if a mixture, and its concentration in moles per liter. $V_L C_L$ and $1 - V_L C_L$ are the volume fractions of the lipid and aqueous phases, respectively. The fractional amount of lipid-bound heme is defined experimentally by $Y = H_b/(H_b + H_f)$, which in terms of Equations 2S and 3S is:

$$Y = \frac{H_m V_L C_L}{H_m V_L C_L + (1 - V_L C_L) H_a} = \frac{K_p V_L C_L}{K_p V_L C_L + 1 - V_L C_L} \tag{4S}$$

In our original analysis, no distinction was made between the inner and outer lipid phases, even for descriptions of the time courses of CO-heme uptake and release. However, the results presented in the Main Text suggest that this was an oversimplification and that binding to the outer and inner lipid layers must be distinguished kinetically. Thus, three steps are involved in heme dissociation and association reactions with liposomes:

$$H_a^o \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H_m^o \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H_m^i \underset{k_{-1}}{\overset{k_{-1}}{\rightleftharpoons}} H_b^i \tag{5S}$$

The superscripts represent the outer, o, and inner, i, layers of the membrane; k_1, k_{-1} represent the rate constants for partitioning between the aqueous, a, and membrane, m, phases; and k_2, k_{-2}, the rate constants for transmembrane flipping. The volume fractions of the four phases in Equation 5S are defined by the concentration of lipid in the suspension, C_L; the partial molar volume of the lipid, V_L; and the outer and inner radii of the vesicle, R_o and R_i

$$\begin{aligned} V_a^o &= 1 - V_L C_L \frac{R_o^3}{R_o^3 - R_i^3} \\ V_m^o &= V_L C_L \frac{R_o^3}{R_o^3 + R_i^3} \\ V_m^i &= V_L C_L \frac{R_i^3}{R_o^3 + R_i^3} \\ V_a^i &= V_L C_L \frac{R_i^3}{R_o^3 - R_i^3} \end{aligned} \tag{6S}$$

V_a^o, V_m^o, V_m^i, *and* V_a^i are the volume fractions of the outer aqueous, outer membrane, inner membrane, and inner aqueous phases, respectively. The fractional amounts of lipid in the outer and inner layers were defined by their relative surface areas assuming a spherical geometry. V_a^i was defined as the ratio of the internal aqueous volume of spherical liposomes to that of the membrane multiplied by the total fractional volume of lipid. V_m^o was then defined as $1 - V_m^o - V_m^i - V_a^i$.

Equation 5S can be simplified since, even for large liposomes, (*diameter = 100 nm*), the internal volume is small, and little or no heme will ever be present in the inner aqueous phase. This can be shown by considering the ratio of the total solution concentration of heme bound in the inner lipid layer to that in the inner aqueous layer at equilibrium, H_b^i/H_f^i, which is given by $Kp\ V_m^i/V_a^i$. The fraction of total internal heme bound in the inner leaflet of the membrane is given by:

$$Y_b^i = \frac{K_p V_m^i / V_a^i}{1 + K_p V_m^i / V_a^i} \tag{7S}$$

In most of our experiments, the vesicle diameter is approximately 100 nm and the thickness of the lecithin membrane is roughly 4.5 nm (*i.e.*, R_o = 50 nm and R_i = 45.5 nm). For these liposomes, V_m^i/V_a^i is 0.148 (Equation 6S) so that even for a lipid mixture which binds heme weakly with $K_p = 1000$ (see Light and Olson, 1990), 99% of the heme on the inside of the vesicle will be bound to the inner membrane monolayer and only 1% will be in the internal aqueous phase. Most of the vesicles studied exhibit K_p values around 10^5, and for these samples, heme would never be found in the inner aqueous phase. Thus, Equation 5S can be reduced to:

$$H_a^o \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H_m^o \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H_m^i$$

$$K_p = \frac{k_1}{k_{-1}};\ K_{1,0} = \frac{k_2}{k_{-2}} = 1.0 \tag{8S}$$

Since there is no evidence to suggest that partitioning into the inner layer is different from that into the outer layer for large vesicles, $K_{1,0}$ is assumed to be 1.0.

Association kinetics - The results in Table I and Figs. 1-6 of the Main Text indicate that k_2, k_{-2} $<< k_1$ *or* k_{-1}. Thus, the initial kinetic phase in CO-heme uptake experiments can be analyzed by considering just the outer lipid layer. At the end of the initial association phase, the fractional amount of CO-heme bound, Y_{fast}, is given by:

$$Y_{fast} = \frac{K_p V_m^o / V_a^o}{1 + K_p V_m^o / V_a^o} \tag{9S}$$

for vesicles with R_o = *50 nm and* R_i = *45.5 nm*, $V_m^o = V_L C_L (0.55)$ *and* $V_a^o = 1 - V_L C_L (4.06)$ (Equation 6S). For the lipids used in this study, $V_L \leq 1.0$ l/mol, $C_L \leq 10^{-3}$M, and thus $V_a^o \approx 1.0$ under all conditions. As a result, Equation 9S reduces to $Y_{fast} \approx K_p V_L C_L (0.55)/(1 + K_p V_L C_L (0.55))$. At the end of the slow phase when the system has achieved true equilibrium, the final fractional amount of heme bound, Y_e, is given by equation 4S. The amplitude of the slow phase is given by $Y_e - Y_{fast}$:

$$Y_e - Y_{fast} = \frac{K_p V_L C_L}{1 + K_p V_L C_L} - \frac{K_p V_L C_L (0.55)}{1 + K_p V_L C_L (0.55)} \tag{10S}$$

and the fractional amount of the slow phase, Y_{slow}, in the overall time course is given by:

$$Y_{slow} = \frac{Y_e - Y_{fast}}{Y_e} = \frac{0.45}{1 + K_p V_L C_L (0.55)} \tag{11S}$$

Thus, the relative magnitude of this phase is both expected and observed to decrease to zero as the lipid phosphate concentration, C_L, is increased (Figure 2B, Main Text).

The rate of heme binding to the outer phospholipid layer can be defined as:

$$\frac{dH_m^o}{dt} = k_1 H_a^o - k_{-1} H_m^o$$

Converting H_a^o and H_m^o to the corresponding total suspension concentrations, H_b^i and H_f^i, and noting that at short times $H_t \approx H_b^i + H_f^i$, the mass balanced differential equation becomes:

$$\frac{dH_b^i}{dt} = \frac{k_1 V_m^o H_f}{V_a^o} - \left[\frac{k_1 V_m^o}{V_a^o} + k_{-1}\right] H_b^i$$

When the rate of transmembrane movement is slow compared to that of uptake and release, this is a simple linear, first order differential equation and predicts that heme binding will exhibit an exponential time course with an observed rate equal to:

$$k_{fast,\ uptake} = k_1 V_L C_L (0.55) + k_{-1} \tag{12S}$$

for vesicles with an outer radius of 50 nm, a bilayer thickness of 4.5 nm, and when $V_a^o \approx 1.0$.

The much slower rate of transmembrane movement can be defined as:

$$\frac{dH_m^i}{dt} = k_2 H_m^o - k_{-2} H_m^i$$

Converting to suspension concentrations using the volume fractions defined in Equation 6S, this becomes:

$$\frac{dH_b^i}{dt} = \frac{k_2 V_m^i H_f^i}{V_m^o} - k_{-2} H_b^i \tag{13S}$$

H_f^o and H_f^i are in equilibrium since k_1 and $k_{-1} >> k_2$ and k_{-2}, and the total CO-heme concentration is given by:

$$H_t = H_f^i + H_a^o + H_b^i$$
$$\text{or } H_t = H_f^i (1 + K_p V_m^o / V_a^o) + H_b^i$$

Substituting $H_a^o = (K_p V_m^o / V_a^o) H_f^i$, and $H_f^i = (H_t - H_b^i)/(1 + K_p V_m^o / V_a^o)$ into equation 13S yields:

$$\frac{dH_b^i}{dt} = \frac{k_2 V_m^i H_t V_a^o}{(1 + K_p V_m^o / V_a^o)} - \left[\frac{k_2 K_p V_m^i / V_a^o}{1 + K_p V_m^o / V_a^o} + k_{-2}\right] H_b^i$$

Thus, heme flipping observed in association experiments will exhibit simple exponential behavior with an observed rate equal to:

$$k_{slow,\ uptake} = \frac{k_2 K_p V_L C_L (0.45)}{1 + K_p V_L C_L (0.55)} + k_{-2} \tag{14S}$$

when R_o = *50 nm* and $V_a^o \approx 1$. The dependence of $k_{slow,\ uptake}$ on lipid phosphate concentration is expected to be small since the rates of transmembrane flipping should be roughly independent of direction (*i.e.*, $K_{1,0} = 1.0$ and $k_2 = k_{-2}$). At $C_L = 0$, the value of $k_{slow,\ uptake}$ will equal k_{-2}; at $C_L \rightarrow \infty$, $k_{slow,\ uptake}$ will equal $1.82\ k_{-2}$. Thus, only an 80% increase in the slow rate would be expected over the entire range of lipid phosphate concentration in heme uptake experiments.

Dissociation kinetics - A slow phase is also observed in heme dissociation time courses in which liposomes containing bound heme are mixed with apomyoglobin (Fig. 3). In these experiments, the initial rapid phase (2-50 s^{-1}) represents the release of heme from the outer portion of the lipid bilayer (Cannon *et al.*, 1984; and Rose *et al.*, 1985). The second phase is much slower and exhibits a rate similar to that observed for the slow phase in CO-heme uptake experiment (Figs. 1-3 and Table I, Main Text). In terms of Equation 8S, this slow phase represents myoglobin formation from CO-heme present initially in the inner monolayer and is limited by the rate of transmembrane flipping (k_{-2}). This process can be represented by:

$$H_m^i \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} H_m^o \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} H_a^o + Gb_a^o \xrightarrow{k_{mb}} Mb_a^o \tag{15S}$$

The rate constants are the same as those in the previous section; k_{mb} is the bimolecular reaction rate for heme binding to apomyoglobin; Gb_a^o *and* Mb_a^o are the apomyoglobin and myoglobin concentrations in the outer aqueous phase; and the reaction of CO-heme with the globin is effectively irreversible (Rose *et al.*, 1985).

If the rate of heme transmembrane movement is slow then the fast phase represents only the efflux of heme initially bound in the outer bilayer and its subsequent rapid reaction with apomyoglobin (*i.e.*, heme flipping can be neglected). At high concentrations of protein, little or no free heme will be present in the aqueous phase, and dH_f^i/dt will equal 0. The rate of myoglobin formation in the initial fast phase will be determined by:

$$\frac{dH_a^o}{dt} = -\frac{k_{-1} k_{Mb} G b_a^o H_m^o}{k_1 V_m^o + k_{Mb} G b_a^o}$$

and

$$k_{fast,\ release} = k_{-1} \left(1 + \frac{k_1 V_L C_L (0.55)}{k_{Mb} G b_a^o}\right) \tag{16S}$$

when R_o = *50 nm and* $V_a^o \approx 1.0$. When $k_{mb} G b^o >> k_1 V_L C_L (0.55)$, this expression reduces to $k_{fast,\ release} = k_{-1}$ (see Rose *et al.*, 1985). The fractional amount of fast phase in release experiments is determined solely by $V_m^o/(V_m^o + V_a^i)$ (0.55 for R_o = *50 nm*) and is independent of $H_t C_L$.

The slow phase observed when apomyoglobin is mixed with heme containing liposomes appears to represent myoglobin formation from CO-heme present initially in the inner monolayer and is limited by the rate of transmembrane flipping. Since the rates k_{-1}, k_1, $k_{mb} G b_a^o >> k_2$, k_{-2}, steady-state assumptions can be made for both H_a^o and H_f^i. Following the similar line of reasoning presented for the derivation of the slow phase seen in the heme association experiments (Equations 13-14S), and using the steady-state assumptions, the rate of heme movement from the inner layer of the outer layer in dissociation experiments is determined by:

$$\frac{dH_1^i}{dt} = -k_{-2}H_1^i\Big/\left[1 + \frac{k_2(k_1V_m^o + k_{M1}Gb^o)}{k_{-1}k_{M1}Gb^o}\right]$$

where the observed rate constant will be:

$$k_{slow,\,release} = k_{-2}\Big/\left[1 + \frac{k_2(k_1V_m^o + k_{M1}Gb^o)}{k_{-1}k_{M1}Gb^o}\right] \qquad (175)$$

At high globin concentrations, this expression reduces to $k_{slow} = k_{-2}/(1 + k_2/k_{-1})$ and since $k_{-1} >> k_2$, $k_{slow,\,release}$ becomes simply k_{-2}, the rate constant for transmembrane movement of CO-heme from the inner to the outer portion of the lipid bilayer. Cannon *et al.*, (1984) derived a similar expression for k_{slow} for this type of experiment using a theory with discrete heme binding sites in the lipid membrane. From these theoretical considerations, the rate of the slow phase observed during the dissociation step is expected to be less that measured for the slow phase during the association reaction at high lipid concentrations (Table I, Main Text).

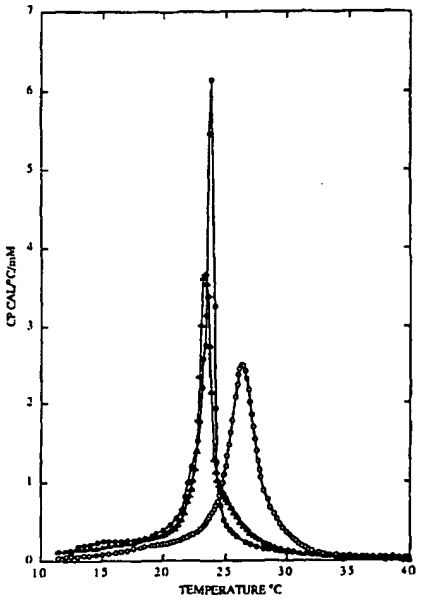

Fig. 15. The heat capacity as a function of temperature for several DMPC liposome compositions. The phase transition temperatures of the DPMC liposomes were measured by differential scanning calorimetry. The points represent the measured values for: DMPC (*closed circles*); DMPC/DCP(9:1) (*open circles*); and DMPC/Hemin(9:1) liposomes (*open triangles*).

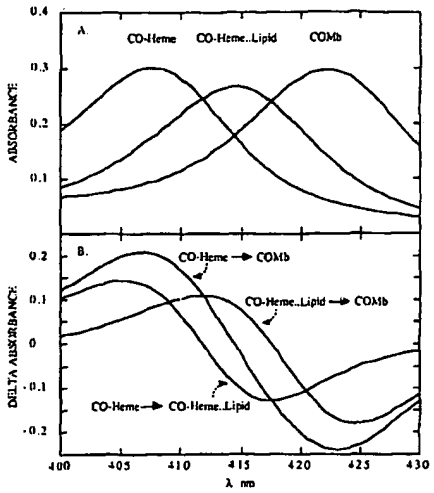

Fig. 25. The spectral characteristics of CO-heme in aqueous buffer, egg lecithin vesicles, or as COMb. A, Spectra for 2 μM heme in the presence of buffer only (CO-Heme), 100 μM egg lecithin (CO-Heme..Lipid), and 5 μM apomyoglobin (COMb), in 0.05M NaCl, 0.05M Tris, pH 8.0, 25°C. *B*, difference spectra for free CO-heme binding to egg lecithin vesicles, free CO-heme binding to apomyoglobin, or the incorporation of membrane-bound CO-heme into apomyoglobin. The delta absorbance values were defined as the absorbance at zero time minus that at infinite time to correspond with the ΔA_λ values obtained in stopped-flow rapid mixing experiments (Figs. 1 and 3, Main Text). Thus, when CO-heme is mixed with apomyoglobin, the absorbance at 420 nm is much greater after the reaction is complete than the initial value, and thus, the total ΔA is a large negative value.